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doi:10.1006/mthe.2000.0037, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Evaluation of the Biodistribution, Persistence, Toxicity, and Potential of Germ-Line Transmission of a Replication-Competent Human Adenovirus Following Intraprostatic Administration in the Mouse

Dell L. Paielli,* Mark S. Wing,* Kenneth R. Rogulski,* Jeff D. Gilbert,* Andy Kolozsvary,* Jae Ho Kim,* Joe Hughes,† Mike Schnell,† Tim Thompson,‡ and Svend O. Freytag*,[1]

*Department of Radiation Oncology, Henry Ford Health System, Detroit, Michigan 48202
†Institute for Human Gene Therapy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104
‡Scott Department of Urology, Baylor College of Medicine, Houston, Texas 77030

Received for publication December 3, 1999, and accepted in revised form February 4, 2000

Adenovirus-mediated gene transfer may hold much promise in the treatment of human cancer. However, concerns regarding vector dissemination beyond the target tissue, particularly with replication-competent viruses, require an evaluation of the persistence of viral infection in collateral tissue and vector-associated toxicities. In addition, for indications such as prostate cancer, the proximity of the point of viral administration to organs of the male reproductive system raises concerns regarding inadvertent germ-line transmission of genes carried by the virus. To address these concerns, the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent adenovirus (Ad5-CD/TK*rep*) following intraprostatic administration in the mouse was examined. Ad5-CD/TK*rep* (10^{10} vp, 5×10^{11} vp/kg) was injected intraprostatically on Day 1 of the study and its presence in the major organs of the male urogenital tract (prostate, testes, seminal vesicles, and urinary bladder) and liver was determined on Days 8 and 29. For comparison, a parallel group of animals was injected with the same dose of a related replication-defective Ad5-FGNR virus. To evaluate germ-line transmission, Ad5-CD/TK*rep*-injected males were mated to females on Days 8 and 29 and resulting embryos were examined for Ad5-CD/TK*rep* viral DNA. Ad5-CD/TK*rep* viral DNA was detected in all major organs of the adult male urogenital tract and liver 7 and 28 Days postinjection. Interestingly, relative to the replication-defective Ad5-FGNR adenovirus, the replication-competent Ad5-CD/TK*rep* virus accumulated to a much greater level (~300-fold) and persisted for a longer period of time in prostate, testes, and liver. This difference could not be explained on the basis of differences in viral infectivity, suggesting that the Ad5-CD/TK*rep* virus may be capable of replicating in mouse tissues *in vivo*. *In vitro* infection of six mouse cell lines representing prostate, testes, and liver demonstrated that the Ad5-CD/TK*rep* virus was indeed capable of replicating in these mouse cell types, albeit with reduced efficiencies relative to human cells. Despite the fact that the Ad5-CD/TK*rep* vector persisted in the adult male gonads and may have replicated *in vivo*, we observed no evidence of germ-line transmission in 149 offspring examined. To evaluate the toxicity of combining Ad5-CD/TK*rep* viral therapy with CD/5-FC and HSV-1 TK/GCV suicide gene therapies as a prerequisite for a human trial, an escalating dose (10^8, 10^9, 10^{10} vp) of Ad5-CD/TK*rep* was administered intraprostatically followed by 7 days of 5-FC and GCV double prodrug therapy. Although the virus persisted in the mouse urogenital tract and liver for up to 28 days postinjection, most of the toxicities observed were expected, minimal, and self-limiting. These results lead us to believe that intraprostatic administration of the Ad5-CD/TK*rep* virus to humans concomitant with double suicide gene therapy will be associated with acceptable toxicities and will not result in vertical transmission of viral-encoded genes through the germ line.

Key Words: human gene therapy; adenovirus; toxicity; germ-line transmission.

[1] To whom correspondence should be addressed at Molecular Biology Research, Henry Ford Health System, One Ford Place, 5D, Detroit, MI 48202-3450. Fax: (313) 876-1950. E-mail: sfreyta1@hfhs.org.

INTRODUCTION

Recombinant adenoviruses are currently the most widely used vectors in human gene therapy trials. Although there are a number of reasons for choosing these agents as gene transfer vectors, their wide use is largely based on their high efficiency of infection. For indications such as cancer, antitumor activity has been observed in a number of preclinical tumor models utilizing a variety of therapeutic genes. Although further improvements will be required before this technology will have broad applicability in the clinic, signs of biologic efficacy in humans are beginning to emerge (1).

As with all gene transfer methods, the use of recombinant adenoviruses as vectors also has potential disadvantages. The major concerns include viral-associated toxicities, wide-spread dissemination of the vector in the patient, and the possibility of vertical transmission of viral-encoded genes through the germ line. Although the latter two issues were evaluated recently in a preclinical model following intravenous administration of a replication-defective adenovirus (2), these issues have not been addressed with replication-competent adenoviruses. Because replication-competent adenoviruses replicate efficiently in human epithelial cells and are cytolytic, their use as gene therapy vectors comes with an increased risk of viral spread, persistence, and damage to normal tissue. Moreover, for indications such as prostate cancer, the close proximity of the point of viral injection to the male reproductive system heightens the concern regarding the possibility of germ-line transmission of viral-encoded genes.

As a prerequisite for a Phase I study in humans, we evaluated the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. We demonstrate here that replication-competent human adenoviruses accumulate to higher levels and persist for longer periods of time *in vivo* relative to replication-defective viruses. Despite this, there was acceptable vector-associated toxicity at doses as high as 5×10^{11} vp/kg and no evidence of germ-line transmission.

MATERIALS AND METHODS

Adenoviruses and cell lines. The Ad5-CD/TK*rep* [previously called FGR (3)] and Ad5-FGNR [previously called Ad5-CDglyTK (3)] adenoviruses containing an *Escherichia coli* cytosine deaminase (CD)/herpes simplex virus type 1 thymidine kinase (HSV-1 TK) fusion gene were propagated in human embryonic kidney (HEK)[2] 293 cells (obtained from Microbix and the American Tissue Culture Collection, ATCC). HEK 293 cells were maintained in minimal essential medium supplemented with 10% fetal calf serum. Virus was purified by isopycnic centrifugation in CsCl gradients followed by dialysis in 10 mM Tris, pH 8.0, 10% (v/v) glycerol for 4 h at 4°C. Virus was stored at −80°C. To determine the concentration of viral particles (vp), an aliquot of the purified virus was disrupted in 0.1% sodium dodecyl sulfate and the optical density measured at 260 nm. It is assumed that 1 OD_{260} is equivalent to 10^{12} vp. The potency of each viral lot, with respect to its cytolytic activity (Ad5-CD/TK*rep*) and ability to confer 5-FC and GCV sensitivity onto cells (Ad5-CD/TK*rep* and Ad5-FGNR), was determined as previously described (3). TM-3, TM-4, H2.35, GC-1spg (obtained from ATCC), and RM-9 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Undifferentiated H2.35 cells were grown at the permissive temperature of 33°C in the presence of 0.2 μM dexamethasone. CUGE cells were maintained in epithelial cell media as previously described (4). *In vitro* viral infections, isolation of low-molecular-weight DNA, and Southern blotting were performed as previously described (3).

Biodistribution and germ-line transmission study. Sterile surgical procedures were used throughout. C57BL/6 male mice (20–22 g) were anesthetized with Nembutal and an incision was made in the lower abdomen. The seminal vesicles and urinary bladder were retracted gently exposing the dorsolateral prostate. Adenovirus (10^{10} vp in 20 μl) was injected into the right dorsolateral lobe of the prostate with the aid of a dissecting microscope (Day 1). At 7 and 28 days postinjection (Days 8 and 29), each male was paired with two females. The next day, half of the males were necropsied and the prostate, testes, urinary bladder, seminal vesicles, and liver were excised and minced on individual clean glass slides using sterile, disposable scalpels. Two animals died prematurely and therefore only eight animals were analyzed at the Day 29 time point. Approximately 250 μl of blood was obtained by supraorbital bleeding. Embryos were harvested from pregnant females at Day 10 of gestation and minced on individual clean glass slides using sterile, disposable scalpels. DNA (both genomic and viral) was isolated using QIAamp spin columns (Qiagen) following the manufacturer's protocol. Surgical instruments used for dissections were thoroughly rinsed between animals with Elminase DNA removal solution (Fisher Scientific) to prevent cross-contamination of viral DNA. As the amount of DNA recovered from some tissues was low, approximate genomic DNA concentrations were determined by electrophoresis of DNA samples on 0.7% agarose–ethidium bromide gels and comparing the intensity of the high-molecular-weight DNA band to genomic DNA standards of known concentration. A Bio-Rad Gel Doc 2000 imager and Quantity One band analysis software (Bio-Rad) were used to compare tissue DNA samples to the standards.

PCR/Southern blot analysis of Ad5-CD/TKrep and Ad5-FGNR viral DNA. The Ad5-CD/TK*rep* genome is 35,116 bp. For 75 ng of a diploid mammalian genome (6×10^9 bp), 1 genomic equivalent (g.e.; 1 copy of viral DNA per diploid genome) is equal to 0.439 pg. A control stock was prepared by spiking 3 μg (in 100 μl) of C57BL/6 mouse (liver) DNA with 1.756 ng of purified Ad5-CD/TK*rep* viral DNA making a 10^2 g.e. stock. Serial dilutions (in 10-fold increments) were prepared using C57BL/6 mouse (liver) DNA (30 μg/ml) as the diluent. Two and one-half microliters (2.5 μl) of each serial dilution contains 75 ng of mouse genomic DNA with varying amounts of the Ad5-CD/TK*rep* viral DNA. The range of controls used in the PCR/Southern blot analyses was 1 to 10^{-4} g.e., which, when using 75 ng of template DNA, is equivalent to 4.39×10^{-13} to 4.39×10^{-17} g of Ad5-CD/TK*rep* viral DNA or 10^4 to 1 viral molecules. A complete standard curve or a 1 g.e. control was run on every gel. The PCR negative control was the complete PCR mixture containing the mouse genomic DNA diluent as template.

Approximately 75 ng of tissue genomic DNA was added to a 25-μl reaction volume containing 12.5 pmol of each primer. The primers amplify a 427-bp sequence in the CD/HSV-1 TK fusion gene which straddles the CD/TK junction region. The 5′ and 3′ primers were 5′-GAGCAGCCAGAAGCCATCGATTAC-3′ and 5′-TCATTACCACCGCCGCGTCCCCGG-3′, respectively. PCR was performed using Ready-to-Go PCR beads (Pharmacia) in a Perkin-Elmer thermocycler using the following conditions: 1 cycle of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min. Ten microliters of the reaction was applied to a 2% agarose gel followed by transfer to Gene Screen Plus (NEN Research Products). The filter was probed with ^{32}P-labeled pCA14-CDglyTK DNA (3) using standard hybridization and washing conditions. All DNA samples were analyzed by PCR for the mouse cytokeratin 18 gene to assess the integrity of the genomic DNA. The 5′ and 3′ primers were 5′-ACCCACACCTACACCCACACTG-3′ and 5′-TACTCTCCTCCCCTGCTCTAAA-3′, respectively. The PCR conditions for the cytokeratin 18 gene were the same

[2] Abbreviations used: HEK, human embryonic kidney; vp, viral particles; m.o.i., multiplicity of infection; g.e., genomic equivalents; CPE, cytopathic effect; PBS, phosphate-buffered saline; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; GCV, ganciclovir; pfu, plaque-forming unit.

as those used for the CD/HSV-1 TK fusion gene. However, because the two primer sets were not compatible, separate reactions had to be performed.

Animal toxicology study. Ninety-three male C57BL/6 mice (20–22 g) were injected intraprostatically with either the Ad5-CD/TK*rep* virus (10^8, 10^9, 10^{10} vp in 5 μl; Groups 1–3 and 6) or saline (Groups 4 and 5) as described above. Two days later, animals that were scheduled to receive prodrugs (Groups 1–4) were given daily intraperitoneal injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) in 1 ml for 7 days (Days 3–9). Control groups received saline. Animals were examined for a number of toxicological parameters: body weight was taken prior to injection and at each necropsy time point; general observations were noted daily; gross observations were at time of necropsy; and at Days 4, 10, and 45, either a partial or full set of tissues was taken for histopathological observations. Clinical chemistries were also examined to determine blood cell levels and liver-specific enzymes at Days 4, 10, and 45. The study (#99-02) was conducted at the University of Pennsylvania's Institute for Human Gene Therapy (Philadelphia, PA). Clinical pathology was performed by Laboratory Corporation of America (Burlington, NC). Histopathology was performed by Experimental Pathology Laboratories Inc. (Herndon, VA). Statistical analyses were conducted by the Department of Biostatistics and Epidemiology at the University of Pennsylvania School of Medicine. The study was performed in compliance with GLP regulations as described in 21 CFR, Part 58. The raw toxicology data will be made available to investigators upon request.

Results

Biodistribution and Persistence Study

The study designs are depicted in Fig. 1. In the biodistribution and germ-line transmission study, a single dose (10^{10} vp, 5×10^{11} vp/kg) of the replication-competent Ad5-CD/TK*rep* vector was injected into the right dorsolateral lobe of the mouse prostate on Day 1 of the study. This dose represents the highest dose level used in the animal toxicology study (described below) and is 40 to 4000 times that (1.25×10^8 to 1.25×10^{10} vp/kg, assuming an 80-kg patient) to be used in a Phase I trial of local recurrence of prostate cancer after definitive radiation therapy (5). Control mice were injected with PBS. The cytolytic Ad5-CD/TK*rep* virus contains a CD/HSV-1 TK fusion gene allowing the use of 5-FC and GCV prodrug therapies to augment its antitumor activity (3, 7). However, because both the CD/5-FC and HSV-1 TK/GCV enzyme/prodrug systems were previously demonstrated to inhibit Ad5-CD/TK*rep* viral replication *in vitro* (3, 7), and 5-FU and GCV impair spermatogenesis (8, 9), prodrugs were withheld in this study to allow for unattenuated viral replication and spread and to maintain male fertility. At 7 (Day 8) and 28 (Day 29) days postinjection, Ad5-CD/TK*rep*-injected males were mated to females after which males were necropsied and various tissues (prostate, testes, urinary bladder, seminal vesicles, liver, blood) were examined for the presence of Ad5-CD/TK*rep* viral DNA using a PCR/Southern blot assay. The Day 8 time point was chosen based on previous studies with a replication-defective adenovirus (ADV/RSV-tk) in which virus was detected in the male urogenital tract 1 week after intraprostatic administration (10). The Day 29 time point was chosen to allow sufficient time for the development of mature sperm from primary spermatocytes, which requires 28 days in the mouse (11). The sensitivity of the PCR/Southern blot assay is at least 1

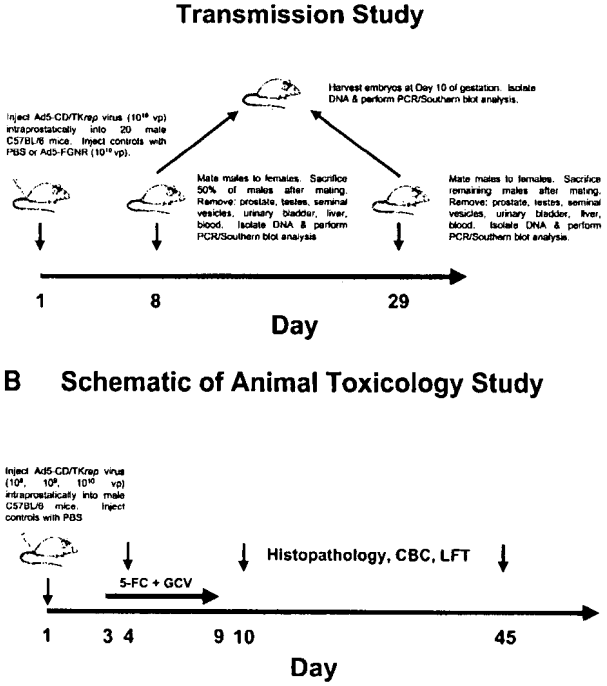

FIG. 1. (A) Schematic of biodistribution and germ-line transmission study. (B) Schematic of toxicology study.

viral copy per 10^4 diploid cells, which, when using 75 ng of genomic DNA as template in the PCR, corresponds to $\sim 4 \times 10^{-17}$ g of Ad5-CD/TK*rep* DNA (35,116 bp) or 1 viral copy (Fig. 2A).

At the Day 8 time point, Ad5-CD/TK*rep* viral DNA was detected in all tissues, except blood, in 100% (10/10) of the mice (Fig. 2B, Table 1). The amount of viral DNA detected was approximately 1 copy/cell in prostate, 0.2 to 0.5 copies/cell in seminal vesicles, urinary bladder, and liver, and ≤0.2 copies/cell in testes. No Ad5-CD/TK*rep* viral DNA was detected in any tissues of the PBS-injected controls.

At the Day 29 time point, the Ad5-CD/TK*rep* virus was found to persist in prostate, testes, and liver of 100% (8/8) of the mice (Fig. 2C, Table 1). Surprisingly, for prostate and liver, the amount of viral DNA detected was essentially identical to that observed at the Day 8 time point. The Ad5-CD/TK*rep* virus also persisted in seminal vesicles and urinary bladder, but at a slightly reduced frequency (75%, 6/8) relative to the Day 8 time point (Fig. 2C, Table 1). Ad5-CD/TK*rep* viral DNA was detected in one of five PBS-injected animals and was likely due to sample contamination.

When combining the results of the Day 8 and 29 time points, Ad5-CD/TK*rep* viral DNA was detected in the prostate of 18 of 18 surviving mice injected with the Ad5-CD/TK*rep* virus, giving an estimated sensitivity of 100%. Nine of 10 (9/10) control animals were negative for Ad5-CD/TK*rep* viral DNA, giving an estimated specificity of 90% and an overall false-positive rate of 10% (1/10).

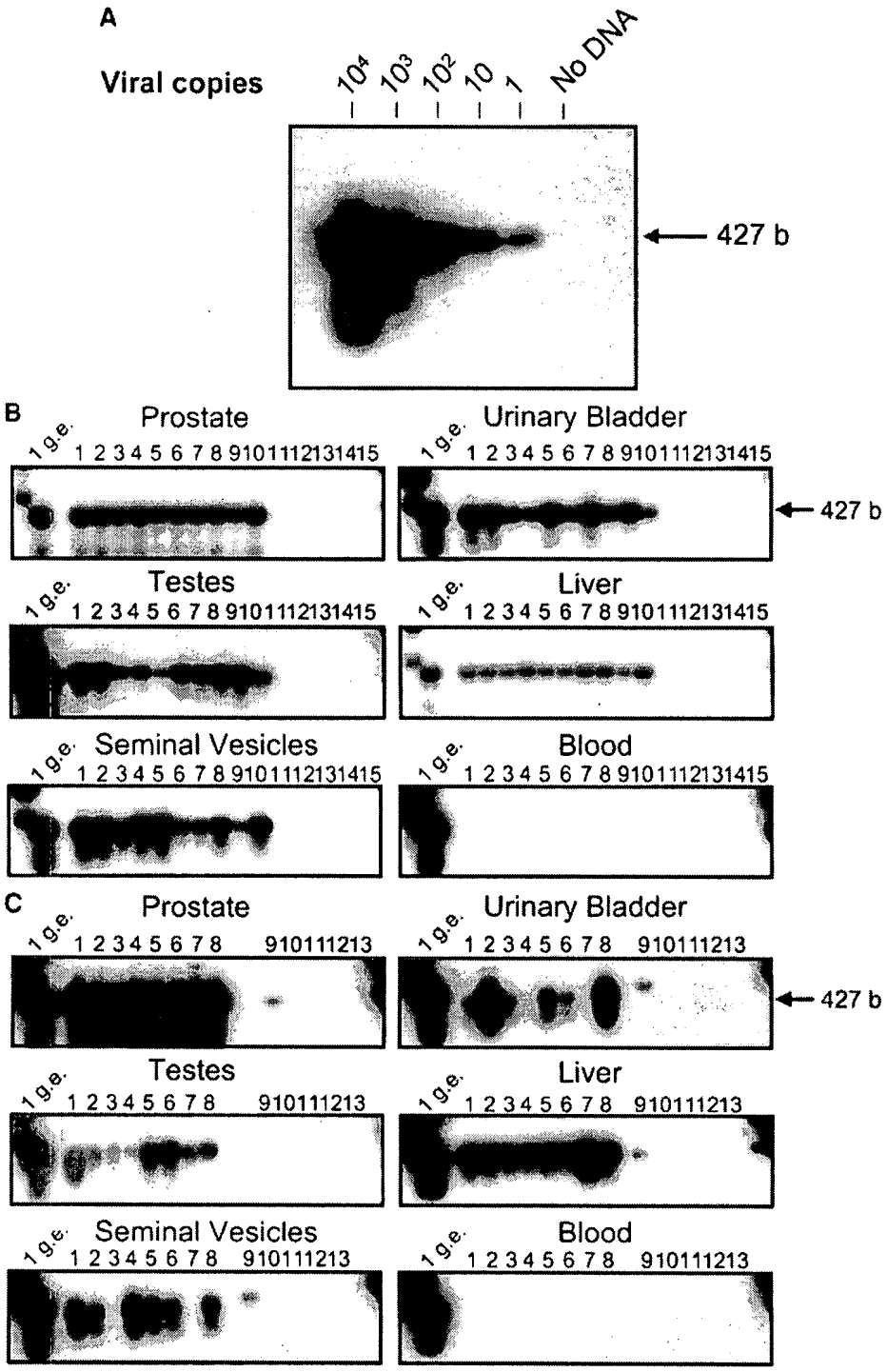

FIG. 2. (A) PCR/Southern blot standard curve. Serial dilutions of purified Ad5-CD/TK*rep* viral DNA were prepared using C57BL/6 mouse liver DNA as the diluent (see Materials and Methods). All PCRs contained 75 ng of genomic DNA. No DNA represents the complete PCR mixture containing the mouse DNA diluent only (no Ad5-CD/TK*rep* viral DNA) as template. Samples were transferred to nitrocellulose and analyzed by Southern blotting. The migration of the expected 427-bp PCR product is indicated. (B) Analysis of Ad5-CD/TK*rep* viral DNA in various mouse tissues on Day 8. Genomic DNA (75 ng) from the indicated tissues were analyzed by the PCR/Southern blot assay. Animals were injected with Ad5-CD/TK*rep* virus (lanes 1–10) and with PBS (lanes 11–15). 1 g.e., 1 genomic equivalent of the Ad5-CD/TK*rep* viral DNA. (C) Analysis of Ad5-CD/TK*rep* viral DNA in various mouse tissues on Day 29. Animals were injected with Ad5-CD/TK*rep* virus (lanes 1–8) and with PBS (lanes 9–14). The prostate, urinary bladder, seminal vesicle, and liver blots were intentionally overexposed to show the presence of Ad5-CD/TK*rep* contamination in one of the control animals (lane 9).

TABLE 1

Summary of Biodistribution and Germ-Line Transmission Studies

	Day 8		Day 29	
	Number positive/ number examined	Viral copies/cell[a]	Number positive/ number examined	Viral copies/cell[a]
Male tissue				
Prostate	10/10	0.5–1.0	8/8	0.5–1.0
Testes	10/10	≤0.2	8/8	≤0.2
Seminal vesicle	10/10	0.2–0.5	6/8	0.2–0.5
Urinary bladder	10/10	0.2–0.5	6/8	0.1–0.5
Liver	10/10	0.2–0.5	8/8	0.2–0.5
Blood	0/10	0	0/8	0
Embryos	0/61	0	0/88	0

[a] Viral copies/cell was estimated by comparing the intensity of each signal to the 1 g.e. control using various autoradiograph exposures and then correcting for differences in exposure time.

Comparison of Replication-Competent Ad5-CD/TKrep and Replication-Defective Ad5-FGNR Adenoviruses

To ascertain whether the level and persistence of replication-competent human adenoviruses differ from the more commonly used replication-defective vectors, a third group of animals was injected intraprostatically with the same dose (10^{10} vp) of a related replication-defective Ad5-FGNR virus (3, 6). Except for the E1A and 19-kDa E1B genes, which are required for adenoviral replication, Ad5-FGNR is identical to Ad5-CD/TK*rep* including the CD/HSV-1 TK fusion gene that is amplified in the PCR. Prostate, testes, and liver were examined for Ad5-FGNR viral DNA.

Rather surprisingly, the amount of Ad5-CD/TK*rep* viral DNA detected in prostate (Fig. 3A, top) and testes and liver (not shown) was at least 100-fold greater than that with the same dose of the replication-defective Ad5-FGNR virus. Whereas 100% of the Ad5-CD/TK*rep*-injected mice contained approximately 1 viral copy/cell in prostate at the Day 8 and 29 time points, Ad5-FGNR-injected animals were estimated to contain <0.01 viral copies/cell. PCR for cytokeratin 18 indicated that the amount and integrity of the input genomic DNA were similar among samples (Fig. 3A, middle). Because the PCR signals generated by the Ad5-FGNR samples were near, or below, background levels, the PCR assays were repeated using a greater amount (500 ng vs 75 ng) of input DNA. One hundred percent (5/5) of the animals scored positive for Ad5-FGNR viral DNA on Day 8 with 80% (4/5) of them having approximately 0.003 viral copies/cell in prostate (Fig. 3A, bottom). In dramatic contrast to the replication-competent Ad5-CD/TK*rep* virus (Fig. 3A, top), the replication-defective Ad5-FGNR virus did not persist *in vivo* as both the frequency and amount of Ad5-FGNR viral DNA decreased significantly at Day 29 relative to the Day 8 time point (Fig. 3A, bottom).

To determine whether this dramatic difference in the amount of viral DNA detected *in vivo* was attributable to differences in viral infectivity, HEK 293 cells were infected *in vitro* with graded m.o.i.'s of the Ad5-CD/TK*rep* and Ad5-FGNR viruses (same lots used in the *in vivo* studies) and the percentage of cells expressing the CD/HSV-1 TK fusion gene was determined by CD immunofluorescence (3). Mouse prostate cells could not be used for these *in vitro* analyses as Ad5-CD/TK*rep*, but not Ad5-FGNR, replicates in such cells (Fig. 4A, below) resulting in markedly higher levels of CD/HSV-1 TK fusion gene expression. When compared side-by-side, this gives the false impression that the infection efficiency of Ad5-CD/TK*rep* is much greater than that of Ad5-FGNR, resulting in a marked underestimation of the true percentage of Ad5-FGNR-infected cells (i.e., in contrast to Ad5-CD/TK*rep*, a significant fraction of Ad5-FGNR-infected cells express the CD/TK fusion protein at near background levels making scoring of many cells equivocal). By contrast, both viruses replicate with equal efficiencies and to high levels in HEK 293 cells making scoring of infected cells unequivocal. Both viruses exhibited essentially identical infection efficiencies *in vitro* at each respective m.o.i. (Fig. 3B), making it unlikely that the dramatic differences observed *in vivo* were due to differences in viral infectivity. Together, these results raised the possibility that the Ad5-CD/TK*rep* virus was capable of replicating in mouse tissues.

To explore this possibility, a variety of mouse cell lines representing prostate (CUGE, RM-9), testes (TM-3, TM-4, GC-1spg), and liver (H2.35) were infected with the Ad5-CD/TK*rep* and Ad5-FGNR viruses and examined for viral DNA replication and cytopathic effects (CPE) *in vitro*. CUGE cells were derived from isolated urogenital sinus epithelium from a 16- to 17-day old mouse fetus and represent precursor cells from which the adult prostatic epithelium is derived (4). RM-9 cells were derived from a mouse prostate carcinoma that was generated *in vivo* by initiation with a *ras* + *myc*-transducing retrovirus and such cell lines have been used extensively as an orthotopic prostate cancer model in the mouse (12, 13). At

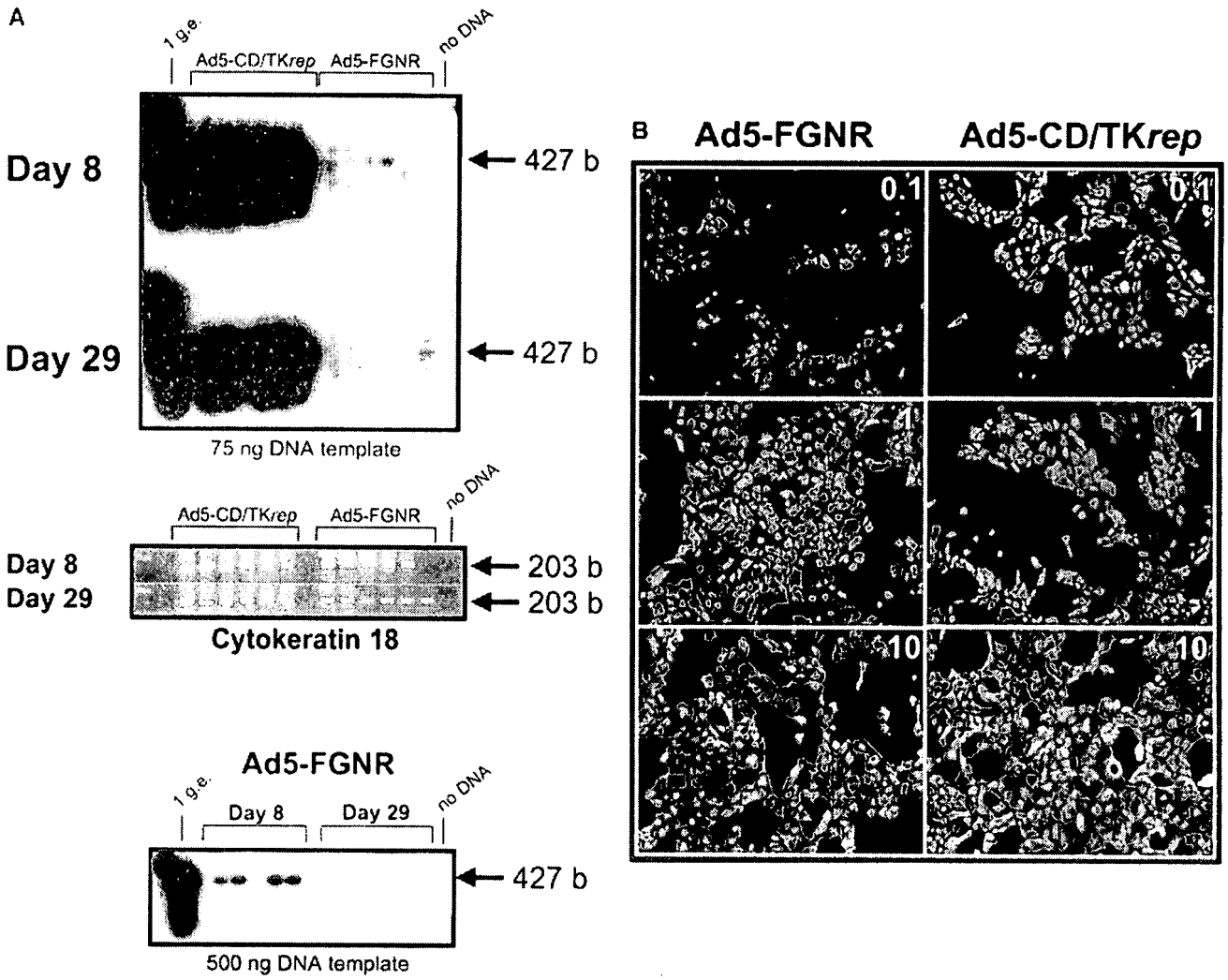

FIG. 3. *In vivo* comparison of replication-competent Ad5-CD/TK*rep* and replication-defective Ad5-FGNR viruses. (A) Twelve animals were injected with 10^{10} vp of Ad5-CD/TK*rep* or Ad5-FGNR as indicated. On Days 8 and 29, animals were necropsied and prostate, testes, and liver were removed for isolation of total DNA. Both Ad5-CD/TK*rep* and Ad5-FGNR contain the CD/HSV-1 TK fusion gene and tissue DNA (75 ng, top; 500 ng, bottom) was examined for the presence of viral DNA using the PCR/Southern blot assay. The intense signal on the left side of the 1 g.e. lane is from the molecular weight markers. Tissue DNAs were also examined for the cytokeratin 18 gene to control for the integrity and amount of input DNA (middle). One Ad5-FGNR-injected animal died prematurely and therefore only 5 animals were analyzed at the Day 8 time point. The migrations of the expected 427-bp (CD/HSV-1 TK fusion gene) and 203-bp (cytokeratin 18 gene) PCR products are indicated. (B) Infection efficiency of Ad5-CD/TK*rep* and Ad5-FGNR viruses *in vitro*. HEK 293 (5×10^4 cells, four-well chamber slides) were infected with graded m.o.i.'s (0, 0.1, 1, 10) of the Ad5-CD/TK*rep* and Ad5-FGNR viruses. Twenty-four hours later, cells were fixed and processed for CD immunofluorescence using a Texas red conjugate (3, 6). Nuclei were stained with DAPI and are blue. Cells that are positive for the CD/TK fusion protein are red. All photographs were taken at 125×. Mock-infected cells (not shown) gave no positive cells.

various times postinfection, low-molecular-weight DNA was isolated and analyzed by Southern blotting for viral DNA. Cells were monitored daily for CPE.

Ad5-CD/TK*rep*, but not Ad5-FGNR, replicated in all mouse cell lines examined, albeit with varying efficiencies (Fig. 4A). Ad5-CD/TK*rep* viral DNA replicated with roughly similar efficiencies in CUGE, RM-9, TM-3, and TM-4 cells reaching a maximum level 2 to 3 days postinfection and somewhat slower in H2.35 and GC-1spg cells reaching a maximum level 4–7 days postinfection (longer time points not shown). The efficiency of Ad5-CD/TK*rep* replication in these six mouse cell lines was estimated to be 3- to 25-fold less than in human DU145 prostate adenocarcinoma cells (Fig. 4A, see legend). The amount of infectious Ad5-CD/TK*rep* virus present in CUGE and DU145 cells 48 h postinfection (preburst) was determined by disrupting cells by three cycles of freeze–thawing and titering the released virus on HEK 293 cells. The amount of infectious virus present in CUGE and DU145 cells differed by 2-fold (not shown), confirming the results of the viral DNA replication studies.

CUGE cells infected with Ad5-CD/TK*rep* demonstrated clear evidence of a cytopathic effect at m.o.i.'s ≥ 10 seventy-two hours postinfection (Fig. 4B). Such cells became rounded and clumped and began detaching from the substratum. By contrast, no CPE (by Day 5) was observed

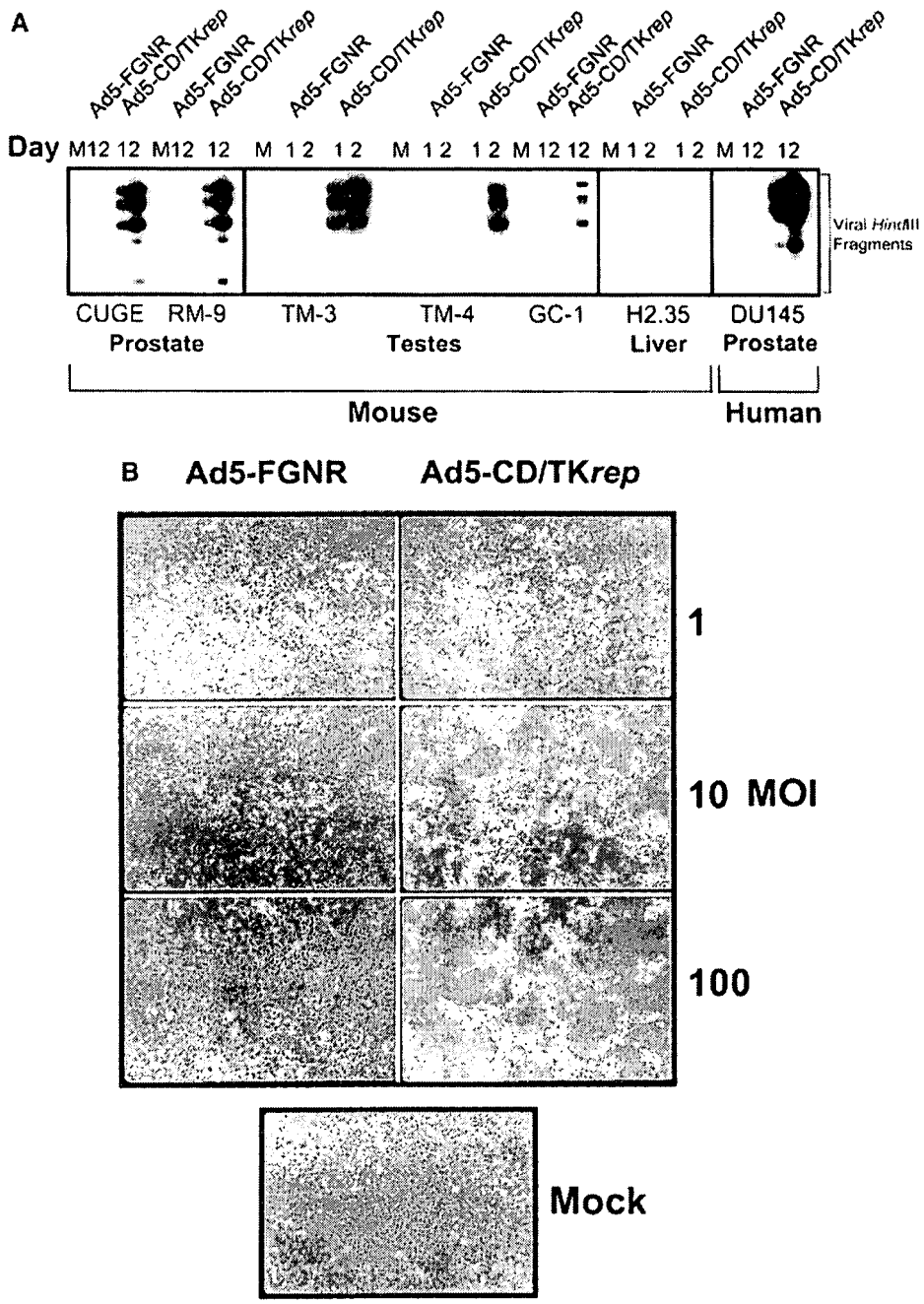

FIG. 4. (A) Replication of Ad5-CD/TK*rep* virus in mouse cells *in vitro*. CUGE, C57BL/6 mouse urogenital sinus epithelial cells (12); RM-9, *ras-myc*-transformed CUGE cells (13); TM-3, BALB/c mouse testicular Leydig cells (14); TM-4, BALB/c mouse testicular Sertoli cells (14), GC-1*spg*, BALB/c mouse SV40-transformed germ cells (15); and H2.35, C57BL/6 mouse SV-40-transformed hepatocytes (16) were infected with Ad5-FGNR or Ad5-CD/TK*rep* at an m.o.i. of 10. On Days 1 and 2 postinfection, cells were harvested and the amount of viral DNA present was determined by Southern blotting (3). DNA was digested with *Hin*dIII. All lanes contain DNA from an equal number of cells. M, mock-infected cells. TM-3, TM-4, H2.25, and DU145 cells were analyzed on the same blot and can be compared directly. CUGE, RM-9, and GC-1spg cells were analyzed on separate blots from the above; however, the number of cells analyzed (10^4), the specific activity of the ^{32}P-labeled probe (10^9 cpm/μg), and the autoradiograph exposure times (4 h) were similar. (B) Cytopathic effect (CPE) of Ad5-FGNR and Ad5-CD/TK*rep* viruses. CUGE cells (10^5 cells/well, 24-well plate) were infected with the Ad5-FGNR and Ad5-CD/TK*rep* viruses using graded (0, 1, 10, 100) m.o.i.'s. Three days later, cells were photographed at 25×.

with mock- or Ad5-FGNR-infected cells up to an m.o.i. of 100. Similar results were obtained with RM-9, TM-3, and H2.35 cells (not shown) and human DU145 cells (3). Together, the results demonstrate that the Ad5-CD/TK*rep* virus can replicate in, and lyse, cells representing tissues of the mouse male urogenital tract (prostate, testes) and liver *in vitro*. When considered with the PCR/Southern blot results (Fig. 3A), they raise the possibility that the

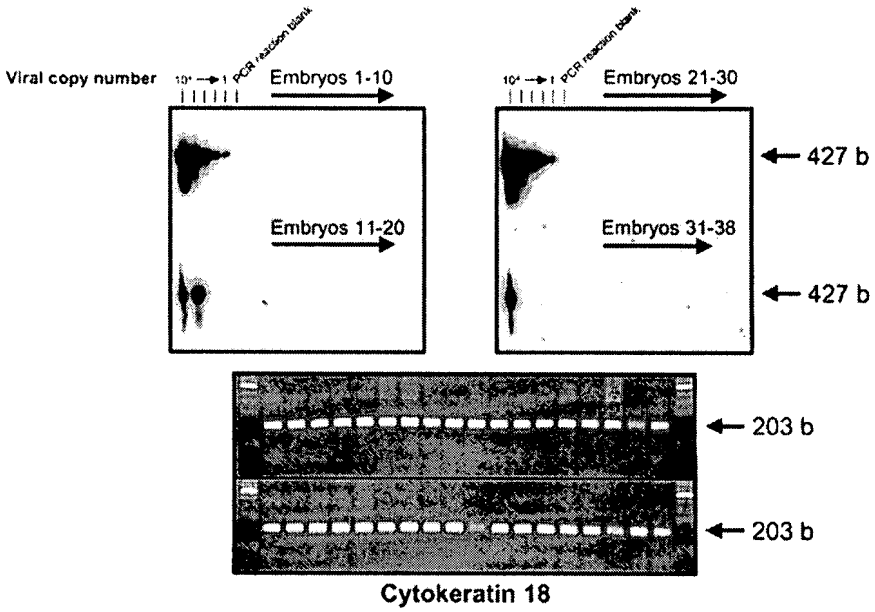

FIG. 5. Analysis of Ad5-CD/TK*rep* viral DNA in embryos from Day 8 mating. Only 38 of 61 embryos examined are shown. Genomic DNA (75 ng) from each embryo was analyzed by the PCR/Southern blot assay. Controls (10^4 to 1 viral copy in log increments) are shown on the upper left of each blot. PCR results for the mouse cytokeratin 18 gene are shown at the bottom. The migrations of the expected 427-bp (CD/HSV-1 TK fusion gene) and 203-bp (cytokeratin 18 gene) PCR products are indicated. All embryo DNAs (149/149) scored positive for cytokeratin 18. Embryos (88) from the Day 29 mating gave identical results.

Ad5-CD/TK*rep* virus may have replicated in these mouse tissues *in vivo*.

Germ-Line Transmission Study

To determine whether Ad5-CD/TK*rep* viral DNA could be transmitted through the germ line following intraprostatic administration, DNA from 149 embryos resulting from the Day 8 and 29 matings was analyzed by the PCR/Southern blot assay. All DNA samples scored positive for the cytokeratin 18 gene and were therefore informative (Fig. 5). No viral DNA was detected in any of the embryos (0/61) from the Day 8 mating despite the fact that Ad5-CD/TK*rep* virus was present in the testes of 100% (10/10) of the animals (Fig. 5, Table 1). Likewise, although the virus persisted in the testes of 100% of the animals at the Day 29 time point, none of the embryos (0/88) was found to contain Ad5-CD/TK*rep* viral DNA (Table 1). Together, the results indicate that the likelihood of germline transmission of Ad5-CD/TK*rep* viral DNA is ≤0.67% following intraprostatic administration.

Toxicology Study

The toxicity of the Ad5-CD/TK*rep* vector without and with 5-FC and GCV double prodrug therapy was examined in the C57BL/6 mouse following intraprostatic injection. The study design is depicted in Fig. 1. The Ad5-CD/TK*rep* virus was injected on Day 1 at three dose levels (1×10^8, 1×10^9, 1×10^{10} vp; Groups 1–3). On a weight basis, this dose range is 40 times that proposed in a Phase I trial of patients with local recurrence of prostate cancer after definitive radiation therapy (5). Two days after viral inoculation, animals were given intraperitoneal injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) for 7 days (Days 3 through 9). Three sets of controls were used to discern both the individual and combined effects of the vector and prodrugs. One group (Group 4) received the prodrug combination and saline in place of the vector, one group (Group 5) received saline in place of both the vector and prodrug combination, and one group (Group 6) received the vector at the highest dose level (10^{10} vp) and saline in place of the prodrug combination. Animals were examined for a number of toxicological parameters: body weight was taken prior to injection and at each necropsy time point; general observations were noted daily; gross observations were noted at time of necropsy; and at Days 4, 10, and 45, either a partial or full set of tissues was taken for histopathological examination. Clinical chemistries were examined to determine blood cell levels and liver-specific enzymes at Days 4, 10, and 45. The results are summarized in Table 2.

The vector was associated with generally minimal clinical signs, except notably three deaths, two in the low-dose group (Group 1) and one in the high-dose group (Group 3). This could have been the result of surgical administration of the vector or the multiple prodrug injections. The lethal dose causing 50% animal death (LD_{50}) was not achieved at 1×10^{10} vp (5×10^{11} vp/kg). There were no significant effects on body weight of any group.

Vector-related toxicities included monocytosis on Day 4 with the highest vector dose (Groups 3 and 6) that resolved by Day 10, mild prostatitis at Days 4 and 10 with the higher vector doses (Groups 2 and 3) with near resolution by Day 45, mild inflammation of the seminal ves-

TABLE 2

Major Clinical and Histopathological Findings in Toxicology Study

Group	Vector	Prodrugs	Day 4	Day 10	Day 45
1	10^8 vp	5-FC + GCV	1 death Inflammation of PR, 0.42 SV, 1.0 UB, ND LV, 0.83 GB, 0.33	0 deaths Inflammation of PR, 0.5 SV, 0.67 UB, 0.67 LV, 1.0 GB, 0.33	1 death Inflammation of PR, 0 SV, 0.17 UB, ND LV, 0.67 GB, 0.67 Testicular deg/atroph
2	10^9 vp	5-FC + GCV	0 deaths Inflammation of PR, 0.83 SV, 1.83 UB, ND LV, 1.17 GB, 0.67	0 deaths Inflammation of PR, 0.92 SV, 1.67 UB, 1.5 LV, 1.0 GB, 0.17	0 deaths Inflammation of PR, 0.33 SV, 0.33 UB, ND LV, 0.67 GB, 0.67 Testicular deg/atroph
3	10^{10} vp	5-FC + GCV	0 deaths Monocytosis Inflammation of PR, 1.17 SV, 1.0 UB, ND LV, 1.17 GB, 0.5	1 death Inflammation of PR, 1.0 SV, 0.4 UB, 0.4 LV, 0.73 GB, 0.4	0 deaths Inflammation of PR, 0.17 SV, 0.5 UB, ND LV, 0.83 GB, 1.17 Testicular deg/atroph
4	None	5-FC + GCV	0 deaths Inflammation of PR, 0.25 SV, 1.33 UB, ND LV, 1.0 GB, 0.67	0 deaths Inflammation of PR, 0 SV, 0.8 UB, 0.6 LV, 0.8 GB, 0.2	0 deaths Inflammation of PR, 0 SV, 0.33 UB, ND LV, 1.0 GB, 0.5 Testicular deg/atroph
5	None	None	0 deaths Inflammation of PR, 0.33 SV, 0.67 UB, ND LV, 0.33 GB, 0.33	0 deaths Inflammation of PR, 0.33 SV, 0.33 UB0.33 LV, 1.0 GB, 0.67	0 deaths Inflammation of PR, 0 SV, 0 UB, ND LV, 1.0 GB, 0
6	10^{10} vp	None	0 deaths Monocytosis Inflammation of PR, 1.0 SV, 0.83 UB, ND LV, 1.17 GB, 0.5	0 deaths Inflammation of PR, ND SV, ND UB, ND LV, ND GB, ND	0 deaths Inflammation of PR, 0.67 SV, 0.33 UB, ND LV, 0.67 GB, 0.83

Note. PR, prostate; SV, seminal veiscles; UB, urinary bladader; LV, liver; GB, gall bladder; deg/atrop, degeneration/atrophy. Numbers represent the average histopathological scores of five to six animals: 1, minimal; 2, mild; 3, moderate; 4, severe. ND, not determined.

icles at Days 4 and 10 that was resolved by Day 45 with the low (Group 1) but not the higher vector doses (Groups 2 and 3), and mild liver and gall bladder inflammation at the highest vector dose (Groups 3 and 6) with the latter exhibiting greater severity when both vector and prodrugs were given. Two animals in the high vector dose plus prodrug group (Group 3) exhibited mild hepatocyte hypertrophy at Day 45. Testicular degeneration and atrophy were observed at Day 45 in all groups that received the prodrug combination (Groups 1–4). Because the incidence and severity were equal among the groups, this effect was attributed to the prodrug combination and not

the vector. The 5-FC and GCV prodrug combination may also affect male potency, as it is associated with a reduced mean litter size (3.0 ± 2) relative to Ad5-CD/TK*rep*- and PBS-injected (10.2 ± 1.2) animals (unpublished results).

Discussion

As a prerequisite for a Phase I trial, we evaluated the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. We demonstrate here that although the Ad5-CD/TK*rep* virus persisted in the adult male urogenital tract, including testes, for up to 28 days postinjection and may have replicated *in vivo*, it did not result in vertical transmission of viral genes in 149 offspring examined (≤0.67%). Moreover, the Ad5-CD/TK*rep* vector was associated with generally minimal toxicity (up to 5×10^{11} vp/kg) when used concomitantly with 5-FC and GCV prodrug therapies.

The persistence and potential of germ-line transmission of a replication-defective adenovirus (H5.001CB*hOTC*) were examined previously in the mouse following intravenous administration (5×10^{10} vp). In that study, H5.001CB*hOTC* viral DNA was detected in both male and female gonads 29 days postinjection but not at 91 and 271 days (2). Despite the presence of viral DNA in the adult gonads at the time of mating (Day 26), there was no evidence of germ-line transmission in 578 offspring examined. The results presented here confirm and extend these findings. Even though the replication-competent Ad5-CD/TK*rep* virus accumulated to a much higher level (~300-fold) in prostate and testes relative to the replication-defective Ad5-FGNR virus and was present in these tissues at the time of mating, we observed no evidence of germ-line transmission, confirming the previous findings of Yu and colleagues (2). It is not known whether the likelihood of viral integration differs between replication-competent and replication-defective adenoviruses. However, the fact that replication-competent adenoviruses express the E1A protein, which induces the host cell to enter S phase, warrants consideration. It is well-established that adeno-associated viruses and retroviruses integrate preferentially into the genome of cells in S phase, although the underlying basis for this phenomenon is unclear (17–19). Thus, although the frequency of adenoviral integration in the host cell genome is believed to be low (20), the fact that replication-competent viruses, such as Ad5-CD/TK*rep*, induce the host cell to enter S phase may enhance the likelihood of integration by providing a more suitable environment for such an event relative to replication-defective viruses. The fact that replication-competent adenoviruses appear to accumulate to a greater level and persist for a longer period of time *in vivo* may also enhance their likelihood of germ-line integration.

In the past, most toxicology studies involving replication-competent human adenoviruses have been performed in the Cotton rat. The sole reason for choosing the Cotton rat over the mouse is that Cotton rats are semi-permissive for adenoviral replication (21). The studies presented here raise an important and controversial issue, namely, is the mouse a valid preclinical model for examining the toxicity and persistence of replication-competent human adenoviruses prior to proceeding to clinical trials in humans? In our opinion, the mouse is as good (or as bad) a model as the Cotton rat for the following reasons.

First, most of the pathologies associated with adenoviral infection are related to viral load, attributable to viral gene expression and the resulting host immune response, and are *unrelated* to virus-induced cytolysis. Intranasal injection of 10^{10} pfu of wild-type Ad5 in the mouse produces the expected pathologies (i.e., pneumonia) without any evidence of viral replication in the lung (22, 23). As expected, the pneumonia is accompanied by infiltration of lymphocytes, monocytes/macrophages, and polymorphonuclear leukocytes and local production of inflammatory cytokines such as tumor necrosis factor α, interleukin-1, and interleukin-6. Although intranasal injection of much lower doses (10^8 pfu) results in both pneumonia and viral replication in the Cotton rat (21), damage to the lung epithelium occurs in the absence of virus-induced cytolysis. At least in rodent models (humans?), the effect of viral replication on the pathological process appears to be manifested through an increase in viral load and gene expression, not virus-induced cytolysis. Thus, it would appear that the mouse is as good (or as bad) a model as the Cotton rat for examining the toxicity of replication-competent human adenoviruses providing that a sufficient viral dose is administered to compensate for the lack of robust viral replication *in vivo*. Whether either model (or any animal model for that matter) can accurately predict their safety profile in humans is unclear. Indeed, the dose-limiting toxicities of replication-competent adenoviruses in humans can be determined only in carefully executed Phase I clinical studies, which are in progress.

Second, we present three lines of circumstantial evidence indicating that the Ad5-CD/TK*rep* virus may have replicated in mouse tissues *in vivo*. Despite the fact that equal amounts (10^{10} vp) of virus were administered, the replication-competent Ad5-CD/TK*rep* virus accumulated to a much higher level (~300-fold) in prostate, testes, and liver relative to the replication-defective Ad5-FGNR virus. These results could not be explained on the basis of differences in viral infectivity. Whereas the amount of Ad5-FGNR viral DNA in these mouse tissues decreased with time as expected for a replication-defective virus, the levels of Ad5-CD/TK*rep* viral DNA at the Day 8 and 29 time points in prostate and liver were essentially equal, indicating that the virus had a means of persisting. Finally, *in vitro* infection of six mouse cell lines representing prostate, testes, and liver demonstrated that Ad5-CD/TK*rep*, but not Ad5-FGNR, was indeed capable of replicating in these mouse tissues, albeit with reduced efficiencies relative to human cells. In four of the six cell lines examined, viral replication resulted in complete cytolysis of the host cell monolayers. When taken together, these results suggest, but do not prove, that the Ad5-CD/TK*rep* virus may

have replicated in these mouse tissues *in vivo*. We have attempted to directly measure Ad5-CD/TK*rep* viral replication *in vivo* by determining the amount of infectious virus present in the mouse prostate at various times postinjection and by labeling viral DNA with bromodeoxyuridine (BUdR). Consistent with the previous observations of others who examined adenovirus replication in the mouse lung (22, 23), both studies failed to provide direct evidence that the Ad5-CD/TK*rep* virus replicated in the mouse prostate *in vivo*. It is worth noting, however, that replication of wild-type Ad5 has been detected in the mouse liver following intravenous administration (24). It is difficult to explain how the Ad5-CD/TK*rep* virus could accumulate to a higher level and persist for a longer period of time than the replication-defective Ad5-FGNR virus without invoking the possibility of viral replication *in vivo*. This possibility is buttressed by our *in vitro* viral replication studies. Although a previous study demonstrated that viral persistence *in vivo* (C57BL/6 mouse) does not require viral replication (25), a side-by-side comparison of the level and persistence of a replication-competent vs replication-defective adenovirus was not examined in that study, as was done here. Therefore, it is not possible to infer from that study whether viral replication potential is a major determinant of viral persistence *in vivo* (the present study would suggest that it is). Thus, when considering the three lines of circumstantial evidence presented here, as well as the *in vivo* observations of others (24), we believe that the replication-competent Ad5-CD/TK*rep* virus may have replicated in the mouse urogenital tract and liver *in vivo* but at a level lower than that observed in the lung of the Cotton rat (and humans).

Despite the fact that the replication-competent Ad5-CD/TKrep adenovirus persisted in the mouse male urogenital tract and liver and may have replicated *in vivo*, most of the observed vector-related toxicities were expected, minimal, and self-limiting up to a viral dose of 5×10^{11} vp/kg (40× the highest dose to be used in our Phase I trial). As expected, monocytosis and inflammation of the prostate and seminal vesicles were observed, but all were generally minimal and largely resolved by the end of the study course (Day 45). Similar results were obtained following intraprostatic administration of a replication-defective ADV/RSV-tk adenovirus (10), although time points beyond Day 7 were not examined in that study. The persistence of the Ad5-CD/TK*rep* virus in liver was unexpected and may be due to replication of the virus in hepatic (ductal) epithelium or continuous shedding of the virus from the prostate. Its persistence in liver is consistent with the histopathological findings of sustained, minimal/mild inflammation of the liver and gall bladder throughout the study course, which was exacerbated somewhat by double (5-FC + GCV) prodrug therapy. This is of concern, as hepatic toxicity can be life-threatening and it has been observed previously in humans following intraprostatic administration of a replication-defective adenovirus (ADV/RSV-tk) concomitant with GCV therapy (1). Thus, the clinical signs of liver/gall bladder toxicity (abnormal liver function tests and blood chemistries, jaundice, and abdominal pain or tenderness) will have to be monitored very carefully in human gene therapy trials involving the replication-competent Ad5-CD/TK*rep* adenovirus, regardless of the route of administration. Although severe testicular degeneration and atrophy were observed in all groups that received double prodrugs, such adverse effects have not been described in humans following GCV (15 mg/kg/day for 14 days) administration (B. Miles, personal communication) and although undesirable are not life-threatening. Whether this adverse event is attributable to the combination of 5-FC and GCV prodrug therapy or the model used (the mouse) is currently unclear and can only be determined in a Phase I clinical study.

Acknowledgments

We thank Drs. Steve Brown and David Monsma for their intellectual contributions. This work was supported by grants from the National Institutes of Health (CA75456, CA64323, and CA75752) and by an award from the RAID Program.

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